Inhibitory effects of dietary calcium on the initial uptake and subsequent retention of heme and nonheme iron in humans: comparisons using an intestinal lavage method[1–4]

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ABSTRACT

Background: Calcium is the only reported dietary inhibitor of both heme- and nonheme-iron absorption. It has been proposed that the 2 forms of iron enter a common pool in the enterocyte and that calcium inhibits the serosal transfer of iron into blood.

Objectives: We aimed to ascertain whether the inhibitory effect of calcium occurs during initial mucosal uptake or during serosal transfer and to compare the serosal transfer of heme and nonheme iron, which should not differ if the 2 forms have entered a common mucosal iron pool.

Design: Whole-gut lavage and whole-body counting were used to measure the initial uptake (8 h) and retention (2 wk) of heme and nonheme iron with and without a calcium supplement (450 mg). Two experiments tested basal meals with low iron bioavailability and 360 mg Ca ($n = 15$) or with high iron bioavailability and 60 mg Ca ($n = 12$).

Results: Added calcium reduced the initial uptake of heme iron by 20%, from 49% to ≈40% from both meals ($P = 0.02$), and reduced the total iron absorbed from the low- and high-bioavailability meals by ≈25% [from 0.033 to 0.025 mg ($P = 0.06$) and from 0.55 to 0.40 mg ($P < 0.01$), respectively]. Calcium did not affect the serosal transfer of either form of iron.

Conclusions: Calcium supplementation reduced heme and total iron without significantly affecting nonheme-iron absorption, regardless of meal bioavailability. Calcium inhibited the initial mucosal uptake rather than the serosal transfer of heme iron. Differences in serosal transfer indicate that heme and nonheme iron did not enter a common absorptive pool within 8 h after a meal. *Am J Clin Nutr* 2005;82:589–97.

KEY WORDS Heme iron, nonheme iron, whole-body counting, whole-gut lavage, mucosal uptake, absorption, retention, serosal transfer, humans, calcium, bioavailability

INTRODUCTION

It has been known since the 1940s that calcium inhibits iron absorption (1). In fact, calcium is the only dietary factor found to inhibit the absorption of both heme and nonheme iron (2–5). Maximal inhibition of nonheme-iron absorption (≈50%) has been shown to occur at a 300-mg dose of calcium (3). This inhibitory effect may present a public health problem because recommendations by the Institute of Medicine (6) and National Institutes of Health (7, 8) for the prevention of osteoporosis have led to widespread use of calcium supplements and fortificants. These practices may exacerbate the effects of marginal iron intakes (9, 10).

The mechanism for the inhibitory effect of calcium on iron absorption is not known. It has been proposed that heme and nonheme iron enter a common mucosal pool and that the inhibition of iron absorption occurs during the serosal transfer process rather than during iron's initial uptake into the enterocyte (4). However, a study showed that a modest amount of calcium (≈120 mg, as cheese) added to a high-iron-bioavailability meal did not reduce either the initial mucosal uptake of nonheme iron or its serosal transfer, as measured by a combination of whole-gut lavage and whole-body scintillation counting (11). It was not clear whether a higher dose of calcium or changes in the bioavailability of the accompanying meal, or both, would result in an inhibition of heme and nonheme forms of iron.

Whereas the initial uptake of nonheme iron was previously estimated by using discriminate analysis of a ^{59}Fe radiotracer and a nonabsorbable radioactive marker excreted in the feces (12, 13) or retained in the body (as measured by whole-body counting) (14, 15), to date no measurement of the initial uptake of heme iron has been reported. Here we describe 2 experiments conducted to test the hypothesis that calcium inhibition of the absorption of heme and nonheme iron occurs during the initial uptake step rather than during the serosal transfer step of the absorptive process. Additional objectives were to compare the 2-wk retention of heme and nonheme iron after their initial entry into the mucosal cell—with the expectation of no difference if the 2 forms of iron have entered a common iron pool in the enterocyte—and to ascertain the relation of serum ferritin to the separate components of iron absorption: mucosal uptake, serosal transfer, and (for nonheme iron) erythrocyte incorporation. To

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accomplish these objectives, we developed a new method to estimate the initial uptake and mucosal transfer of heme and nonheme iron by combining whole-gut lavage and whole-body counting procedures.

SUBJECTS AND METHODS

General protocol

The effect of supplemental calcium (450 mg as citrate) on the initial mucosal uptake, subsequent serosal transfer, and retention of heme and nonheme iron was measured in 2 experiments differing in basal test meals. Experiment A tested a meal with low iron bioavailability and moderate calcium content ($n = 15$), and Experiment B tested a basal meal with high iron bioavailability and low calcium content ($n = 12$). Healthy participants in each experiment consumed the respective test meal twice, once with and once without a calcium supplement (450 mg as citrate), in random order and separated by 6 wk. Each meal contained both heme (^{55}Fe, emitter of low-energy X-rays) and nonheme (^{59}Fe, emitter of γ-rays) radiotracers. The entire gut contents were purged 8 h later with an orally administered lavage solution of polyethylene glycol. This 8-h period for initial uptake was chosen arbitrarily to allow for passage of chyme through the upper intestinal tract (16) with minimal sloughing of mucosal cells (life span: 2–3 d) (17). Initial mucosal uptake was estimated from the isotope retention at 8 h, and absorption was estimated from retention at 2 wk. The difference was taken to represent serosal transfer of iron from the enterocyte. In this report, unless otherwise specified, the terms absorption and retention are used interchangeably to refer to the retention of the isotopes in the body 2 wk after the test meal.

Subjects

Participants were recruited through public advertising. Those selected were aged ≥21 y, had no apparent underlying disease or routine use of medications, had normal hemoglobin (≥12 g/L for women, ≥14 g/L for men), had serum concentrations of ferritin < 450 μg/L, had not been pregnant in the past year, were not breastfeeding, had not donated blood in the past 2 y, and had not used iron supplements in the past 6 mo. Participants agreed to discontinue all nutrient supplements when they applied, generally 6–12 wk before the study.

The participants gave written informed consent. The study was approved for human subjects by the University of North Dakota Radioactive Drug Research Committee and its Institutional Review Board and by the US Department of Agriculture Human Studies Review and Radiological Safety Committee. Subject characteristics for both experiments are described in **Table 1**.

TABLE 1
Subject characteristics by experiment

	Experiment A (n = 8M, 7F)	Experiment B (n = 6M, 6F)
Age (y)[1]	38 ± 12 (21–53)	37 ± 10 (21–48)
BMI (kg/m^2)[1]	27 ± 4 (20–34)	27 ± 5 (22–35)
Serum ferritin (μg/L)[2]	32 (4–195) [9, 112]	55 (13–219) [24, 125]
Serum iron (μmol/L)[1]	66 ± 28 (17–120)	65 ± 25 (28–121)
Total-iron-binding capacity (μmol/L)[1]	319 ± 38 (254–378)	283 ± 49 (245–418)
Transferrin saturation (%)[1]	21 ± 10 (5–43)	24 ± 11 (7–49)

[1] x ± SD; range in parentheses.
[2] Geometric x; range in parentheses; −SD and +SD in brackets.

Test meals

For Experiment A, the test meal was low in iron bioavailability and had moderate calcium content; it consisted of a wheat muffin (60 g), eggs (50 g), Canadian bacon (15 g), shredded-wheat cereal (28 g), margarine (5 g), white sugar (5 g), milk (227 g, 2% fat), and tea (1 g dry instant powder). It contained 360 mg calcium, 0.04 mg heme iron, 3.9 mg total iron (by analysis), and 1871 mg phytate, calculated (18).

For Experiment B, the test meal was high in iron bioavailability and had low calcium content; it was patterned after the meal described by Lynch et al (19) and consisted of ground beef (90 g), a bun (53 g), French fries (68 g), apple juice (240 g), and tomato ketchup (40 g). It contained 60 mg calcium, 0.7 mg heme iron, 4.3 mg total iron (by analysis), and 462 mg of phytate (calculated from published analyses of similar foods; 18).

The participants consumed the weighed test meals quantitatively at the research center. They fasted for ≥10 h before and 8 h after the test meals. A carbonated, caffeine-free, sugar-containing beverage was allowed at the midpoint of the 8-h fast to alleviate any discomfort due to fasting. Water was also allowed.

The radio tracers ^{55}Fe (19 kBq as rabbit hemoglobin) and ^{59}Fe (37 kBq as $FeCl_3$) and dysprosium (1.0 mg as $DyCl_30 \cdot 6H_2O$; Sigma, St. Louis, MO), a poorly absorbed, rare earth metal used as a fecal marker (20), were added to the meat portion of each test meal. The meal was briefly reheated in a microwave oven before service. The ^{55}Fe and ^{59}Fe isotopes were purchased from NEN Life Science Products (Boston, MA). Radiolabeled hemoglobin was obtained by intravenously injecting 74 MBq (2 mCi) of ^{55}Fe into an iron-deficient, pathogen-free rabbit, exsanguinating the animal 2 wk later, and removing Rollet's stroma by lysing and centrifugation (21). The specific activity of the final preparation was 0.585 kBq/μg iron. The amounts of iron added to each test meal as a result of labeling with ^{55}Fe and ^{59}Fe were ≈32 and ≈0.4 μg, respectively.

Lavage procedure and isotope measurements

After each test meal and the subsequent 8-h fast, the participants were admitted to a private room in a metabolic ward. To purge their gastrointestinal contents, the participants drank 4 L of an isomotic/isotonic polyethylene glycol solution at a rate of 240 mL every 10 min (GoLYTELY; Braintree Laboratories Inc, Braintree, MA). They collected the entire lavage effluent in plastic bags until 1 h after completely drinking the lavage solution.

Retention of ^{59}Fe was ascertained with the use of a custom-made whole-body counter, described elsewhere (22). Participants underwent whole-body counting before the meals (background), 1–3 h after the meals (initial dose), after the intestinal lavage (initial mucosal uptake), and 2 wk later (absorption). All human isotope measurements were corrected for background measurements and physical decay.

For the isotope analyses of the stools, the lavage effluent samples of each participant were lyophilized, weighed, and homogenized, and all samples were pooled. From this pooled sample, nine 1.5-g aliquots were weighed and placed into 50-mL

teflon digestion tubes (Cole Palmer Instrument Company, Vernon Hills, IL). The influence of the stool matrix on isotope recovery was assessed for each aliquotted stool sample, by adding 19 kBq ^{55}Fe to 3 aliquots and 37 kBq ^{59}Fe to another 3 aliquots. Isotope recovery was further assessed by digesting triplicate samples of each isotope without stools. The samples were digested under conditions of intermediate heating with nitric acid, followed by the addition of 30% hydrogen peroxide. Isotope concentrations were measured in a liquid scintillation counter (Tri-Carb 1600 TR; Packard, Meriden, CT) and expressed as fractions of the administered radioisotopes. Isotope recovery from digested samples was 101 ± 7% and 91 ± 6% for ^{55}Fe and ^{59}Fe, respectively.

The ratio of the isotopes measured in the lavage excreta, which accounted for nearly all of the gut solids (by inspection), was highly (R^2 = 0.99; data not shown) correlated with the ratio measured in representative excreta samples after correction for recovery of the unabsorbed dysprosium fecal marker. Although this correlation suggests that a total stool collection would not be necessary in future investigations, the current investigation used the data from the total stool collection, without further using the dysprosium measurements.

On day 15 (2 wk) after the meals, we measured the radioisotope concentrations in blood (23). These measurements, together with estimates of total blood volume based on sex, body height, and weight (24, 25), were used to determine the fraction of administered ^{55}Fe and ^{59}Fe in blood.

Calculations of initial mucosal uptake, absorption, serosal transfer, and red blood cell incorporation of nonheme iron

The mucosal uptake and absorption of nonheme iron were determined from whole-body counting of ^{59}Fe after the lavage and 2 wk after the meal, respectively, according to the following equations:

Fractional mucosal uptake of nonheme iron

$$= {}^{59}\mathrm{Fe}_{\mathrm{wbc,lavage}}/{}^{59}\mathrm{Fe}_{\mathrm{wbc,dose}} \quad (1)$$

Fractional absorption of nonheme iron

$$= {}^{59}\mathrm{Fe}_{\mathrm{wbc,2wk}}/{}^{59}\mathrm{Fe}_{\mathrm{wbc,dose}} \quad (2)$$

where: $^{59}\mathrm{Fe}_{\mathrm{wbc,lavage}}$ = ^{59}Fe by whole-body counting after the lavage procedure, $^{59}\mathrm{Fe}_{\mathrm{wbc,dose}}$ = ^{59}Fe by whole-body counting 1–3 h after the test meal, and $^{59}\mathrm{Fe}_{\mathrm{wbc,2wk}}$ = ^{59}Fe by whole-body counting 2 wk after the test meal.

The mucosal uptake of heme iron was calculated by applying the ratios of the 2 isotopes in the dose and in the lavage excreta to the whole-body count after the lavage according to the following equation:

Fractional mucosal uptake of heme iron

$$= 1 - [(^{55}\mathrm{Fe}/^{59}\mathrm{Fe})_{\mathrm{lavage}} \cdot (^{59}\mathrm{Fe}/^{55}\mathrm{Fe})_{\mathrm{dose}} \cdot (1 - {}^{59}\mathrm{Fe}_{\mathrm{wbc,lavage}}/{}^{59}\mathrm{Fe}_{\mathrm{wbc,dose}})] \quad (3)$$

where: $(^{55}\mathrm{Fe}/^{59}\mathrm{Fe})_{\mathrm{lavage}}$ = the ratio of isotopes in the lavage excreta, and $(^{59}\mathrm{Fe}/^{55}\mathrm{Fe})_{\mathrm{dose}}$ = the ratio of isotopes in the administered dose. (*See* Appendix A for derivation of the equation.)

The absorption of heme iron was calculated by applying the ratio of the 2 isotopes in the blood after 2 wk to the nonheme-iron absorption measurement according to the following equation:

Fractional absorption of heme Fe

$$= \mathrm{frac}^{55}\mathrm{Fe}_{\mathrm{blood,2wk}}/\mathrm{frac}^{59}\mathrm{Fe}_{\mathrm{blood,2wk}} \cdot {}^{59}\mathrm{Fe}_{\mathrm{wbc,2wk}}/{}^{59}\mathrm{Fe}_{\mathrm{wbc,dose}} \quad (4)$$

where: $\mathrm{frac}^{55}\mathrm{Fe}_{\mathrm{blood,2wk}}$ = the fraction of administered ^{55}Fe in blood after 2 wk, and $\mathrm{frac}^{59}\mathrm{Fe}_{\mathrm{blood,2wk}}$ = the fraction of administered ^{59}Fe in blood after 2 wk.

A serosal transfer index was calculated for each form of iron, respectively, as the fraction of the initial mucosal uptake that was absorbed, according to the following equation:

Serosal transfer index of each form of iron

$$= \text{fractional absorption/fractional mucosal uptake} \quad (5)$$

Finally, the percentage of the absorbed nonheme iron that was incorporated into blood was calculated from the blood and whole-body ^{59}Fe retention measurements 2 wk after the meals, according to the following equation:

RBC incorporation as percentage of absorbed nonheme iron

$$= \mathrm{frac}^{59}\mathrm{Fe}_{\mathrm{blood,2wk}} \cdot {}^{59}\mathrm{Fe}_{\mathrm{wbc,dose}}/{}^{59}\mathrm{Fe}_{\mathrm{wbc,2wk}} \cdot 100 \quad (6)$$

Although the data are not presented in this report, iron absorption can also be estimated by using only blood measurements, with the assumption that 80% of the newly absorbed isotope is incorporated into blood. For nonheme-iron absorption, results from blood isotope measurements were strongly and significantly correlated with those from the whole-body counting measurements in this study (R^2 = 0.98, $P < 0.0001$) and in previous studies (26, 27). For heme-iron absorption, the results were less strongly correlated (R^2 = 0.56, $P < 0.0001$) when results from blood isotope measurements were compared with those from whole-body counting (as in equation *4* above). The latter method assumes that the heme- and nonheme-iron labels are similarly incorporated into erythrocytes.

Diet analysis

The iron content of the test meals was measured after acid digestion (28) by using inductively coupled argon plasma emission spectrophotometry. Mean (±SD) analyses of standard reference materials from the National Institute of Standards and Technology were 95 ± 9% of certified values. Nonheme iron in the test meal was extracted to minimize pigment breakdown (29) and analyzed by the same digestion and inductively coupled argon plasma methods. Heme iron in the test meal was calculated as the difference between total and nonheme iron. Our previous analyses indicated that cooking procedures (baking and brief reheating by microwave) did not affect the heme-iron content of meat.

Other analyses

Hemoglobin and hematocrit were measured with the use of a Cell-Dyne 3500 System (Abbott Laboratories, Abbott Park, IL). Serum iron was measured colorimetrically by using a Cobas Fara

TABLE 2
Components of iron retention from the meal with low iron bioavailability and moderate calcium content, with or without the addition of a 450-mg Ca supplement (experiment A)[1]

	Without calcium	With calcium	*P*
Nonheme-iron mucosal uptake (%)	2.1 (1.6, 2.7)[2]	2.4 (1.9, 3.1)	NS
Nonheme-iron mucosal uptake (mg)	0.077 (0.067, 0.089)	0.091 (0.078, 0.104)	NS
Nonheme-iron absorption (%)	0.5 (0.4, 0.6)	0.4 (0.3, 0.5)	NS
Nonheme-iron absorption (mg)	0.018 (0.016, 0.020)	0.014 (0.012, 0.016)	NS
Nonheme-iron serosal transfer index[3]	0.33 ± 0.05[4]	0.28 ± 0.08	NS
Heme-iron mucosal uptake (%)	49 (45, 52)	39 (37, 42)	0.02
Heme-iron mucosal uptake (mg)	0.020 (0.019, 0.021)	0.016 (0.015, 0.017)	0.02
Heme-iron absorption (%)	30 (27, 32)	22 (19, 25)	0.06
Heme-iron absorption (mg)	0.011 (0.010, 0.011)	0.009 (0.008, 0.009)	0.06
Heme-iron serosal transfer index[3]	0.57 ± 0.05	0.62 ± 0.05	NS
Total iron retention (mg)	0.033 (0.030, 0.037)	0.025 (0.022, 0.027)	0.06
Erythrocyte incorporation (% of absorbed nonheme iron)	74 ± 6	83 ± 7	NS

[1] The low-iron-bioavailability basal meal contained 0.04 mg heme Fe, 3.9 mg nonheme Fe, and 360 mg Ca. The addition of 450 mg Ca was tested in random order ($n = 15$). Significance was defined as $P < 0.05$.
[2] Geometric x; −SE, +SE in parentheses (all such values).
[3] For each form of iron, the serosal transfer index was calculated as the iron retention at 2 wk divided by the initial iron uptake at 8 h, expressed here as a fraction.
[4] $x \pm$ SE (all such values).

Chemistry Analyzer (Hoffman-LaRoche Inc, Nutley, NJ) with a commercial chromagen (Ferene; Raichem Division of Hemagen Diagnostics, San Diego, CA). Iron-binding capacity was similarly measured after the addition of a known amount of ferrous iron to the serum sample under alkaline conditions. Percentage transferrin saturation was calculated from serum iron and total-iron-binding capacity. Serum ferritin was measured by using an immunoassay kit (Abbott Laboratories). In an effort to detect increases in serum ferritin related to inflammation, C-reactive protein was measured by using nephelometry (Behring Diagnostics Inc, Westwood, MA).

Statistical analysis

The data on the initial mucosal uptake and retention of heme and nonheme iron and the serum ferritin concentrations were logarithmically transformed. For the transformed data, geometric means are reported. The effects of calcium were evaluated by using repeated-measures analysis of variance and SAS software (version 9.1.2; SAS Institute, Cary, NC; 30), which indicated no influence of treatment sequence. Differences between the initial mucosal uptake and retention of heme and nonheme iron were measured by using *t* tests (30). Simple linear regression analyses (30) were used to assess additional relations between variables.

RESULTS

Initial mucosal uptake and retention of nonheme and heme iron

Experiment A

Calcium added to the low-iron-bioavailability, moderate-calcium meal did not affect the relatively low initial mucosal uptake (2.1%) and absorption (0.5%) of nonheme iron (**Table 2**). Approximately one-third of the nonheme iron initially taken up by the enterocytes was subsequently absorbed and retained, and this serosal transfer also was unaffected by the addition of calcium (*see* Table 2). In contrast, added calcium reduced the mucosal uptake of heme iron from this meal from 49% to 39% ($P = 0.02$; Table 2) and reduced the absorption of heme iron from 30% to 22% (P for trend = 0.06; Table 2). More than half of the heme iron that entered the intestinal cells was transferred to the body (serosal transfer index: 0.57; Table 2), and this transfer was unaffected by the addition of calcium. The supplemental calcium tended to further reduce the relatively low amount of total iron absorbed from the low-iron-bioavailability meal by ≈25%, from 0.033 to 0.025 mg ($P = 0.06$; Table 2).

Experiment B

The addition of calcium to the high-iron-bioavailability, low-calcium meal did not significantly reduce the mucosal uptake of nonheme iron (13% and 10% for the meal without and the meal with calcium, respectively; NS; **Table 3**) but tended to reduce the absorption of nonheme iron from 8% to 6% ($P = 0.07$) (Table 3). The serosal transfer of nonheme iron from this meal was relatively high, with more than two-thirds of the nonheme iron initially taken up into the intestinal cells subsequently being retained, but this serosal transfer was not affected by the addition of calcium (Table 3). As with the low-iron-bioavailability meal (Table 2), calcium added to the high-iron-bioavailability meal reduced the initial mucosal uptake of heme iron (from 49% to 40%; $P = 0.02$; Table 3). It also reduced heme-iron absorption (from 22% to 16%; $P = 0.01$; Table 3). With the high-iron-bioavailability meal, approximately one-half of the heme iron taken up by the intestinal cells was transferred to the body (serosal transfer index: 0.48), and this transfer was not affected by the addition of calcium (Table 3). Calcium supplementation of this meal significantly reduced the total amount of iron absorbed by ≈27%, from 0.55 to 0.40 mg ($P = 0.01$; Table 3).

Total iron absorption with the high-iron-bioavailability, low-calcium meal was roughly 15 times that with the low-iron-bioavailability, high-calcium meal. Although statistical comparisons were not made between the 2 experiments, the fractional

TABLE 3
Components of iron retention from the meal with high iron bioavailability and low calcium content, with or without the addition of a 450-mg Ca supplement (experiment B)[1]

	Without calcium	With calcium	*p*
Nonheme-iron mucosal uptake (%)	13 (10, 16)[2]	10 (9, 11)	NS
Nonheme-iron mucosal uptake (mg)	0.551 (0.483, 0.627)	0.408 (0.358, 0.465)	NS
Nonheme-iron absorption (%)	8 (7, 11)	6 (5, 8)	0.07
Nonheme-iron absorption (mg)	0.356 (0.325, 0.390)	0.274 (0.025, 0.300)	0.07
Nonheme-iron serosal transfer index[3]	0.70 ± 0.07[4]	0.72 ± 0.06	NS
Heme-iron mucosal uptake (%)	49 (45, 53)	40 (36, 43)	0.02
Heme-iron mucosal uptake (mg)	0.351 (0.334, 0.369)	0.285 (0.271, 0.300)	0.02
Heme-iron absorption (%)	22 (20, 25)	16 (14, 19)	0.01
Heme-iron absorption (mg)	0.156 (0.146, 0.166)	0.118 (0.110, 0.125)	0.01
Heme-iron serosal transfer index[3]	0.48 ± 0.06	0.44 ± 0.06	NS
Total iron retention (mg)	0.55 (0.52, 0.59)	0.40 (0.38, 0.43)	0.01
Erythrocyte incorporation (% of absorbed nonheme iron)	80 ± 6	82 ± 6	NS

[1] The high-iron-bioavailability basal meal contained 0.7 mg heme Fe, 3.6 mg nonheme Fe, and 60 mg Ca. The addition of 450 mg Ca was tested in random order ($n = 12$). Significance was defined as $P < 0.05$.
[2] Geometric x; −SE, +SE in parentheses (all such values).
[3] For each form of iron, the serosal transfer index was calculated as the iron retention at 2 wk divided by the initial iron uptake at 8 h, expressed here as a fraction.
[4] $x \pm$ SE (all such values).

mucosal uptake, serosal transfer, and absorption of nonheme iron were apparently greater with the high-iron-bioavailability diet than with the low-iron-bioavailability diet (Tables 2 and 3). The mucosal uptake of heme iron did not differ substantially between the 2 studies, but heme-iron absorption was somewhat less with the high- than with the low-iron-bioavailability meal, which reflects a somewhat smaller heme-iron serosal transfer.

Absorption values for the heme and nonheme forms of iron were compared within each experiment. For both low- and high-iron-bioavailability meals (experiments A and B, respectively), heme iron was taken up and absorbed more efficiently than nonheme iron ($P < 0.0001$ for both, paired t tests; data not shown). However, with the low-iron-bioavailability meal, the fractional serosal transfer for nonheme iron was approximately half as efficient as that for heme iron (0.30 and 0.60, respectively; $P < 0.0001$; experiment A). In contrast, with the high-iron-bioavailability meal, this transfer was more efficient for nonheme than for heme iron (0.71 and 0.46, respectively; $P < 0.0001$, paired t tests; data not shown in tables; experiment B).

Correlations of serum ferritin with iron-absorption variables

Body iron stores, as indicated by serum ferritin concentrations, correlated inversely with nonheme-iron absorption and total iron absorption in both studies, in either the absence or presence of added calcium (**Table 4**; **Figure 1**). However, serum ferritin was not associated with heme-iron absorption and was not consistently associated with the measures of initial uptake or serosal transfer for either form of iron (Table 4).

Erythrocyte incorporation of absorbed iron

Two weeks after the test meal, ≈80% of the newly absorbed nonheme iron was incorporated into the erythrocytes of subjects in both experiments, independent of calcium treatment (Tables 2 and 3). This erythrocyte incorporation did not significantly correlate with serum ferritin in either experiment (Table 4).

DISCUSSION

In this study, we developed a new method of measuring the initial mucosal uptake of heme iron by using a combination of whole-gut lavage and whole-body counting, and we applied the

TABLE 4
Correlation of iron-absorption values with serum ferritin[1]

	Without calcium		With calcium	
	r	*P*	*r*	*P*
Experiment A ($n = 15$)				
Nonheme iron				
Initial mucosal uptake (%)	−0.19	NS	−0.49	0.06
Absorption, 2-wk retention (%)	−0.51	0.05	−0.58	0.02
Serosal transfer index	−0.18	NS	−0.26	NS
Erythrocyte incorporation (% of absorbed iron)	−0.35	NS	−0.44	NS
Heme iron				
Initial mucosal uptake (%)	0.41	NS	0.10	NS
Absorption, 2-wk retention (%)	−0.13	NS	−0.16	NS
Serosal transfer index (% of mucosal uptake)	−0.35	NS	−0.24	NS
Total iron (mg)	−0.53	0.04	−0.58	0.02
Experiment B ($n = 12$)				
Nonheme iron				
Initial mucosal uptake (%)	−0.71	0.01	−0.53	0.08
Absorption, 2-wk retention (%)	−0.85	0.001	−0.78	0.003
Serosal transfer index	−0.45	NS	−0.81	0.002
Erythrocyte incorporation (% of absorbed iron)	−0.53	NS	−0.54	0.07
Heme iron				
Initial mucosal uptake (%)	−0.34	NS	0.15	NS
Absorption, 2-wk retention (%)	−0.17	NS	−0.37	NS
Serosal transfer index (% of mucosal uptake)	0.06	NS	−0.50	NS
Total absorbed iron (mg)	−0.86	0.001	−0.73	0.01

[1] All data except those for the serosal transfer index and erythrocyte incorporation were log transformed before the regression analysis. Experiment A, low-iron-bioavailability, moderate-calcium meal; experiment B, high-iron-bioavailability, low-calcium meal. Significance was defined as $P < 0.05$.

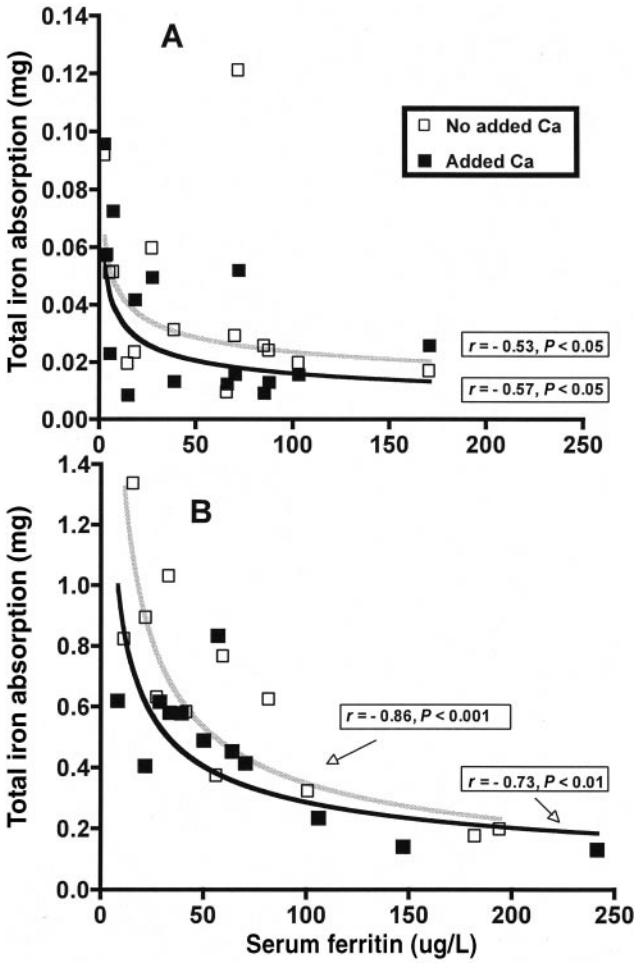

FIGURE 1. Whole-body retention of total iron from a low-iron-bioavailability meal (A; $n = 15$) and a high-iron-bioavailability meal (B; $n = 12$), each consumed with (■) and without (□) a calcium supplement (450 mg, as citrate). The whole-body retention of total iron correlated inversely with serum ferritin in both experiments regardless of whether a calcium supplement was consumed with the meal.

method to a comparison of the effect of supplemental calcium on the initial uptake and the serosal transfer components of heme- and nonheme-iron absorption. Added calcium consistently inhibited heme-iron absorption by reducing its initial mucosal uptake, and it did this without affecting heme iron's serosal transfer. Although calcium reduced heme-iron and total iron absorption, its effect on nonheme-iron absorption was not significant in these experiments.

Hallberg et al (3, 31) found that calcium inhibited both nonheme- and heme-iron absorption, and they proposed that, because the 2 iron forms likely have different apical mucosal receptors, calcium inhibition likely occurred in the final steps of transport from the mucosal cell to plasma, after the 2 forms of iron had entered a common cellular iron pool. This proposed mechanism was not supported by our findings, however, because the addition of calcium did not alter the serosal transfer index of either form of iron (Tables 2 and 3). Nevertheless, calcium inhibition could occur through the inhibition of iron transport during the initial entry of iron into the mucosal cell, even with differences in apical receptors for heme and nonheme iron. In rats, dietary calcium inhibited iron absorption by delaying the entry of nonheme iron into the microvilli of intestinal epithelial cells (32). Similarly, in a cell culture model, calcium was shown to inhibit the transport of ferrous iron by the divalent metal transporter-1 (DMT-1, formerly called DCT-1) (33), an important receptor for nonheme-iron uptake into the enterocyte (34). DMT-1 could also be involved in the calcium inhibition of heme-iron uptake. Although the mechanism of heme-iron uptake is not completely understood, studies suggest that heme iron enters the enterocyte as an intact iron-protoporphyrin structure (35) through the brush border membrane by the process of endocytosis (36, 37). If the release of iron by the action of heme oxygenase, as described by Raffin et al (38), occurs within the resulting tubulovesicle, DMT-1 could be involved in the further transfer of the released iron across this tubulovesicular membrane to the intercellular space. This possible scenario requires further investigation: the mechanism for the calcium inhibition of heme-iron uptake is still unknown. The control of iron absorption also likely involves the regulation of serosal transfer, and this process uses the copper-dependent ferroxidase hephaestin (39) or the serosal transport protein ferroportin (40) or both; in turn, ferroportin is posttranslationally controlled by the apparent regulatory peptide hepcidin (40–42). However, the current results indicate that calcium inhibition of heme-iron absorption occurs during mucosal uptake, not during serosal transfer.

Our findings of significant differences in the fractional serosal transfer of heme and nonheme iron (Tables 2 and 3) did not support the hypothesis that these iron species enter into a common pool within the enterocyte, at least not within 8 h of meal consumption. As observed in dogs, heme iron is absorbed through a subcellular route that is, at least initially, distinct from the path for nonheme iron (36) and that may involve different transfer rates. The recent description of a heme export protein that is expressed in the intestine suggests that heme iron may also be at least partially absorbed in an intact form (43). Therefore, the differences in the serosal transfer index observed in the current study may be explained by differences in the localization or the rate of transfer (or both) of the 2 forms of iron in the enterocyte.

We previously reported, on the basis of an inverse association with serum ferritin, that the initial uptake of nonheme iron is the primary point at which its absorption is controlled (11). Those previous findings are consistent with the inverse correlation coefficients observed in the current study (Table 4), although not all of them were significant. The effect of iron status on the biological control of nonheme-iron retention was evident, because the absorption of nonheme iron was inversely correlated to serum ferritin under all test conditions (Table 4). Others have found that the initial mucosal uptake was the rate-limiting step in nonheme-iron absorption (44) and that it was inversely related to iron status (14, 15).

It is difficult to evaluate which component of heme-iron absorption serves as its primary control point, because neither the uptake nor the absorption of heme iron correlated significantly with serum ferritin concentrations, regardless of the iron bioavailability of the meal (Table 4). However, a significant inverse relation between heme-iron absorption and iron status has been repeatedly shown (19, 27, 28, 45–47), and this suggests some biological control of heme-iron absorption.

Calcium inhibition of nonheme-iron absorption has been shown repeatedly (2–4, 48, 49) but inconsistently (50, 51). It is not clear why calcium did not significantly reduce nonheme-iron absorption in the current study. The 450-mg calcium dose, a

common dose in supplements, was chosen to be sufficient for maximal inhibition of nonheme-iron absorption. It has been shown that calcium inhibits nonheme-iron absorption by 40–60% at doses between 165 and 300 mg, and that there is no further inhibition as the dose exceeds 300 mg (3). The relatively high (360 mg) basal calcium content of the current low-iron-bioavailability meal may have minimized any calcium inhibition of nonheme iron that was already poorly absorbed (experiment A; Table 2). However, Cook et al (2) observed calcium inhibition (55%) of nonheme-iron absorption from a similar low-iron-bioavailability, high-calcium meal. In the current study, the use of calcium in the citrate form may have influenced the results; in a study by Cook et al (2), the citrate form was less inhibitory than was calcium carbonate or calcium phosphate when tested with a high-iron-bioavailability meal. However, the current results included a nonsignificant decrease (≈20%, Table 2) in nonheme-iron absorption with the low-iron-bioavailability meal and a nearly significant decrease (≈25%; $P = 0.07$; Table 3) with the high-iron-bioavailability meal. Because these nonheme iron results contributed to reductions in the total amount of iron absorbed (0.008 and 0.15 mg for experiments A and B, respectively) that far exceeded the reductions observed with heme iron alone (0.002 and 0.038 mg), we concluded that the nonsignificant decrease in nonheme-iron absorption also contributed to the overall reduction in iron absorption.

The initial uptake of heme iron from the meals in the current study was 49%, irrespective of iron bioavailability and basal calcium content (Tables 2 and 3). The addition of 450 mg calcium inhibited this initial uptake by ≈20% and inhibited heme-iron absorption by ≈27% with both meals (Tables 2 and 3). This suggests that the inhibiting effect of calcium on heme-iron absorption was not influenced by the basal calcium concentration (360 mg in experiment A and 60 mg in experiment B). The magnitude of calcium inhibition was similar for the 2 forms of iron (a nonsignificant or marginally significant inhibition of 20–25% for nonheme and a significant inhibition of 27% for heme), which is consistent with another report of similar but greater inhibition (≈40–50%) with the 2 forms of iron (31).

Some have questioned the use of single test meals, rather than whole diets, in studies of iron absorption. The nonsignificant effect of calcium on nonheme-iron absorption in one study of whole diets may have been the result of incomplete control of the experimental diets (52). Much as was seen in the current single-meal study, when weighed diets were tested under controlled conditions, reductions of 10–31% with ≈800 mg calcium from different sources were not significant with a 4-d diet (51). In contrast, another whole-diet study showed that a redistribution of calcium sources (milk and cheese) from breakfast and an evening snack only to all meals of a 10-d diet, including lunches and dinners with iron from meat, poultry, or fish, significantly reduced total iron absorption by ≈25% (53).

The current study and most of those cited above evaluated only the short-term effect of calcium on iron absorption. Although nonheme-iron absorption partially adapts to changes in iron bioavailability (26, 54) and intake (27), differences in iron bioavailability do not change iron status within several weeks (26, 54), and in fact it may require several years for the iron status to change (50). Consistent with this possibility, calcium supplementation for several months did not affect serum ferritin in premenopausal women (55, 56), lactating women (57), or healthy adults of both sexes (49). Nonetheless, the long-term use of dietary calcium salts in supplements and fortificants may further increase the risk of iron deficiency in women who are having difficulty in meeting their iron requirements.

In summary, whole-gut lavage and whole-body counting procedures were successfully used in a new method to provide the first estimates of the initial mucosal uptake of heme iron from 2 test meals with different iron bioavailability. Consumption of a calcium supplement reduced the total iron absorbed, primarily by reducing the initial uptake of heme iron. Differences in the transfer of heme and nonheme iron from the mucosal cell into the bloodstream suggest that the 2 forms of iron did not join a common pool immediately after uptake into the mucosal cells.

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All coauthors contributed to the design and implementation of the research. CAZ planned and implemented the radioiron analyses of blood and urine and the related quality controls. Both ZKR and JRH critically interpreted the data and revised the manuscript. ZKR wrote the original draft of the manuscript. The authors were employees of the US Department of Agriculture–Agricultural Research Service. None of the authors had any personal or financial conflict of interest.

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APPENDIX A

The equations in this Appendix denote the calculation of the fractional mucosal uptake (MU) of heme iron ($MU\ ^{55}Fe$) using the analyzed ratios of ^{55}Fe and ^{59}Fe in the administered dose and in the lavage excreta, as measured by digestion procedures, and the mucosal uptake of ^{59}Fe, as measured by whole-body counting.

The ratio of isotopes in the lavage excreta may be written as the ratio of the difference between the amount of administered dose and the amount taken up into the body (MU) for each isotope, as in the following equation:

$$(^{55}Fe/^{59}Fe)_{lavage} = [^{55}Fe_{dose} - {}^{55}Fe_{dose}\,(MU^{55}Fe)] \Big/ [^{59}Fe_{dose} - [^{59}Fe_{dose}(MU^{59}Fe)] \quad (A1)$$

where: $(^{55}Fe/^{59}Fe)_{lavage}$ = the ratio of isotopes in the lavage excreta, $^{55}Fe_{dose}$ = the amount of ^{55}Fe in the dose, $^{59}Fe_{dose}$ = the amount of ^{59}Fe in the dose, $MU^{55}Fe$ = the fractional mucosal

uptake of ^{55}Fe, $^{55}Fe_{dose}(MU^{55}Fe)$ = the amount of mucosal uptake of ^{55}Fe, $MU^{59}Fe$ = the fractional mucosal uptake of ^{59}Fe, and $^{59}Fe_{dose}(MU^{59}Fe)$ = the amount of mucosal uptake of ^{59}Fe. The next step is solving equation *A1* for $MU^{55}Fe$:

$$MU^{55}Fe = 1 - [(^{55}Fe/^{59}Fe)_{lavage} \cdot (^{59}Fe/^{55}Fe)_{dose} \cdot (1 - MU^{59}Fe)] \quad (A2)$$

where: $(^{59}Fe/^{55}Fe)_{dose}$ = the ratio of isotopes in the administered dose. The fractional MU of ^{59}Fe ($MU^{59}Fe$) may be estimated by the ratio of the ^{59}Fe in the body immediately after the lavage procedure ($^{59}Fe_{wbc,\ lavage}$) to the ^{59}Fe present 1–3 h after the test meal dosing ($^{59}Fe_{wbc,\ dose}$), as shown in the following equation:

$$MU^{59}Fe = {}^{59}Fe_{wbc,lavage}/{}^{59}Fe_{wbc,dose} \quad (A3)$$

and, by substituting the last portion of equation *A3* for $MU^{59}Fe$ in equation *A2*, the following equation is formed:

$$MU^{55}Fe = 1 - [(^{55}Fe/^{59}Fe)_{lavage} \cdot (^{59}Fe/^{55}Fe)_{dose} \cdot (1 - {}^{59}Fe_{wbc,lavage}/{}^{59}Fe_{wbc,dose})] \quad (A4)$$

which is the same as equation *3*, given earlier in the text.